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Previews

A CRY in the Night

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CRY1 is essential for normal circadian clock function, but its transcriptional regulation by the clock has not been considered an important feature for its function. However, reporting in *Cell*, Ukai-Tadenuma et al. (2011) now show that rhythmic *Cry1* expression in the early night is critical for clock function.

In mammals, circadian clocks are present in most cells. These drive local, approximately 24 hr rhythms in the expression of thousands of genes in anticipation of the predictable environmental changes associated with day and night. The clock mechanism involves a network of interlocked transcriptional feedback loops that drive rhythmic expression of genes integral to clock function (i.e., "core clock" genes) and "output" genes that facilitate rhythms in biological processes (Reppert and Weaver, 2002; Takahashi et al., 2008). So far, three transcriptional loops appear to be involved, each primarily centered on a different DNA enhancer element—the E/E′-box, the REV-ERB/ROR element (RRE), or the D-box (Figure 1). The E/E′-box transcriptional loop is essential for circadian clock function and is thought to be the primary timekeeping mechanism. In this loop, heterodimers of the E/E′-box activators CLOCK/NPAS2 and BMAL1/BMAL2 drive expression of their own transcriptional repressors PER1, PER2, CRY1, and CRY2. After an essential time delay, these repressors form a multimeric complex that translocates to the nucleus to inhibit the activity of CLOCK:BMAL1. Similarly, activators in the RRE loop regulate repressors and vice versa. The importance of the D-box loop has not been as fully explored. All three of these transcriptional loops are heavily interconnected (Ueda et al., 2005). Factors in the E-box loop regulate D-box activators (e.g., DBP), which, in turn, drive expression of RRE activators (RORs). E-box factors also regulate the RRE repressor, REV-ERBα, which drives rhythmic expression of E-box activators and the D-box repressor, NFIL3. Importantly, using combinations of these elements, it is possible to direct expression of reporter constructs to virtually any time of the day. These loops represent motifs in a network architecture (Figure 1) that generates clock function and robust rhythms in circadian gene expression at all phases of the day.

Despite its pervasiveness, the specific timing, or phase, of clock-gene expression has not been fully explored in many of these models. One of the remaining mysteries is the regulation of *Cry1*, which peaks in expression in the early night, a phase not readily explained solely by E-box regulation. Initial discovery of two RREs in the first intron of *Cry1* led to the hypothesis that its regulation may be the product of both modules (Etchegaray et al., 2003). However, stoichiometry studies of clock proteins suggested that CRY1 abundance is not rate-limiting for clock function (Lee et al., 2011, 2001; Fan et al., 2007). Using an elegant synthetic biology approach to study how these different enhancer elements contribute to *Cry1* expression at the right time of day, Ukai-Tadenuma et al. (2011) now find that rhythmic *Cry1* expression during its proper phase is vital for clock function.

Ukai-Tadenuma et al. (2011) first identified a functional E′-box that overlaps with a functional D-box in the *Cry1* promoter and confirmed the presence of the RREs in the first intron of the *Cry1* gene. Next they showed that these elements are functional and rhythmically bound in vivo by their cognate transcription factors BMAL1, DBP, and REVERBα. Interestingly, these three proteins were rhythmically bound to their elements in nearly the same phase, but antiphase to the *Cry1* mRNA rhythm. This suggests that *Cry1* expression may be predominantly dictated by derepression (i.e., removal of REVERBα) at the RREs. To test the relative contribution of the E′-box/D-box (combined) and RRE elements in determining the phase of *Cry1* expression, the authors placed various combinations of each element upstream of a luciferase reporter and determined the phase of the rhythm produced by these constructs in rhythmic fibroblasts. E′-box/D-box only reporters drove luciferase rhythms that peaked in the afternoon, whereas reporters containing just *Cry1*'s intronic RREs drove luciferase rhythms that peaked in the late night. However, addition of *Cry1*'s intronic RREs to the E′-box/D-box reporter produced luciferase rhythms that peaked in the early night, a time between that elicited by either elements on their own. Furthermore, modulating the strength of the E′-box/D-boxes and RREs relative to one another produced changes in the phase of expression that could be predicted using simple vector addition models: strengthening E′-box/D-box elements advanced the phase toward afternoon phase, whereas strengthening RREs delayed the phase toward the late night. Overall, these results strongly suggest that proper timing of *Cry1* expression is regulated in a combinatorial fashion, involving activation at E/E′-box and D-boxes that is delayed by repression/derepression at RREs. This complex architecture defines a mode of transcriptional regulation within the clock that drives gene expression to peak in the early night.

To assess the functional significance of rhythmic and/or early night expression of *Cry1*, Ukai-Tadenuma et al. (2011) tested whether these characteristics of its regulation were necessary to rescue arrhythmicity of *Cry1/Cry2* null mutant fibroblasts. Using this cell-based genetic complementation approach, they found that neither constitutive expression, nor expression of *Cry1* in the morning/afternoon via E′-and D-boxes, could restore rhythms to mutant

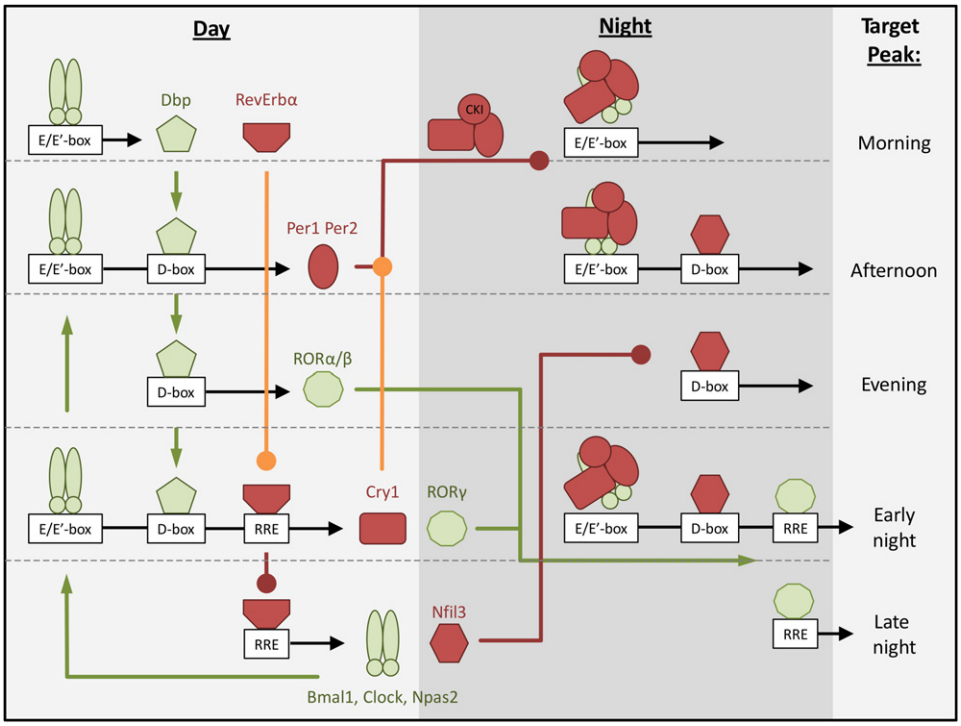

Figure 1. Principles of Circadian Clock Design
Wiring diagram depicting five transcriptional regulation pathways within the mammalian circadian clock. Transcriptional activators are in light green, and their pathways are depicted by lines ending with arrowheads. Repressors are in dark red, and their pathways are depicted by lines ending with circles. The transcriptional delay of *Cry1* to the early evening imposed by REVERBα repression at RREs serves as a critical "delay" built into the clock for accurate ~24 hr timekeeping (orange lines, Ukai-Tadenuma et al., 2011).

cells. Remarkably, they could only rescue *Cry1* deficiency when expression of *Cry1* was delayed to the early night, a phase determined by the combined activity at E/E′-boxes, D-boxes, and RREs. Furthermore, shifts in the balance between the E′-box/D-box activation strength and the RRE repression strength that alter the phase of the *Cry1* rhythm also alter the period of the overall clock rhythm. Importantly, Ukai-Tadenuma et al. (2011) showed that constructs that failed to rescue CRY1 deficiency expressed *Cry1* at levels comparable to those constructs that did rescue. Thus, these results strongly argue that not only is the *Cry1* mRNA expression rhythm an essential component of overall clock function, but that its delayed phase dictated by the combinatorial activity at the three enhancer elements appears to be critical for clock function.

These findings are not necessarily at odds with previous studies that suggested regulation of *Cry1* may not be important for clock function (Fan et al., 2007; Lee et al., 2001, 2011). CRY1 biochemical function is clearly important, but so is the timing of its expression. Perhaps the timing of CRY1 protein accumulation has a substantial role in stabilizing PER proteins as they accumulate, or maybe newly synthesized CRY1 regulates nuclear entry of the PER:CRY complex. Regardless, determining the mechanism by which nighttime *Cry1* expression is required for oscillator function will be an important and challenging goal. For example, to approach this in vivo, a flexible system to regulate *Cry1* gene expression to multiple phases followed by behavioral and molecular analysis in the mouse would be required. Moreover, the importance of transcriptional regulation for many other clock factors has not yet been fully explored. Indeed, the results presented by Ukai-Tadenuma et al. (2011) highlight the fact that the circadian clock still has many hidden complexities that have yet to be elucidated.

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